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(54) **Klebsiella oxytoca No. 19-1 and a process for producing alpha-cyclodextrin.**

(57) The present invention relates to a new strain of *Klebsiella oxytoca* No. 19-1 separated from soil digesting starch and producing  $\alpha$ -cyclodextrin with a very high selectivity and also to a process for producing  $\alpha$ -cyclodextrin exclusively in high yield from starch by use of cyclomalto-dextrin glucanotransferase produced by the strain.

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## BACKGROUND OF THE INVENTION

The present invention relates to a new strain of *Klebsiella oxytoca* No. 19-1 capable of producing  $\alpha$ -cyclodextrin exclusively from starch, and to a process for producing  $\alpha$ -cyclodextrin from starch with cyclodextrin glucanotransferase produced by the strain.

Cyclodextrins are cyclic oligosaccharides composed of glucose units and have known as host molecules which have torus in the molecule capable of forming inclusion complexes with various kinds of organic compounds, mainly with hydrophobic compounds.

Thus, cyclodextrins have been widely used in the fields of foods, chemicals, pharmaceuticals and agricultural chemistry and so on, in view of the following advantages:

- Modification of physical and chemical properties, for example, stabilization of volatile materials, protection against oxidation and UV degradation, increment in solubility of water-insoluble materials, shifting of colors, and deodorization.
- Emulsification of fats and oils.
- Acceleration and control of reaction, and improvement of yield.

Cyclodextrins are composed of 6, 7 or 8 glucose residues which are bound by  $\alpha$ -1,4-glucosidic linkage, and called  $\alpha$ -,  $\beta$ - or  $\gamma$ -cyclodextrin depending on the number of carbon residue, 6, 7 or 8, respectively. The existence of branched cyclodextrins connected by  $\alpha$ -1,6-glucosidic linkage has also been reported and the property of them is quite different from each other.

Extensive application of  $\alpha$ -cyclodextrin among them, is expected in the food and medical industries, since  $\alpha$ -cyclodextrin has higher solubility in water than  $\beta$ -cyclodextrin and is hardly digested by  $\alpha$ -amylase of saliva and intestine, thereby classified as a non-metabolized dietary fiber which is not harmful to human body and digested by some Bacteroides when adsorbed into the body.

In conventional processes for producing  $\alpha$ -cyclodextrin, starch as a substrate is reacted with an enzyme preparation selected from culture medium or various steps of purification which is obtained by cultivating microorganisms capable of producing cyclodextrin glucanotransferase, and  $\alpha$ -cyclodextrin is then obtained from reaction mixtures by treatment of organic solvents or complicated processes, for example, gel filtration.

Up to now, various strains producing cyclodextrin have been public, particularly, there are typical strains producing  $\alpha$ -cyclodextrin such as *Bacillus macerans*, *Bacillus stearothermophilus* and *Klebsiella pneumoniae*.

However, since the strains known as microor-

ganisms producing  $\alpha$ -cyclodextrin do not produce only  $\alpha$ -cyclodextrin but produce simultaneously another types of cyclodextrins, it is still required to complicated purification process in preparation of  $\alpha$ -cyclodextrin in high degree of purity.

Meanwhile, it was reported that the production of  $\alpha$ -cyclodextrin was also increased by the presence of water-immiscible organic solvent like *n*-decyl alcohol and detergent like sodium dodecyl sulfate in the reaction mixture.

However, the use of toxic solvents or detergents seems to be undesirable since  $\alpha$ -cyclodextrin produced using these are prohibited in food processing, and consequently it has confronted a problem requiring more complicated purification steps to remove the added chemical compounds.

Recently, Japanese Patent Laid-opened No. 89-225493 discloses a method of producing  $\alpha$ -cyclodextrin from *Klebsiella pneumoniae* subsp. *pneumoniae*  $\alpha$ -CD, without any treatment of toxic solvents or detergents to promote the production of  $\alpha$ -cyclodextrin. However, this method is still required to improve the process since there are some disadvantages in this method in point of concomitant production of large amounts of  $\beta$ -cyclodextrin, low production yield of  $\alpha$ -cyclodextrin (10g/L), and prolonged reaction time of 3 days, although this method does not produce branched cyclodextrins.

## SUMMARY OF INVENTION

The present invention aims to provide a new strain producing  $\alpha$ -cyclodextrin exclusively from starch and also to provide process for producing  $\alpha$ -cyclodextrin with cyclodextrin glucanotransferase produced by the strain.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention relates to a new strain, *Klebsiella oxytoca* No. 19-1 having the ability to produce  $\alpha$ -cyclodextrin with a very high selectivity from starch.

And, the present invention includes a process producing  $\alpha$ -cyclodextrin exclusively by reacting starch as a substrate with an enzyme preparation selected from cultured medium or various steps purification which is obtained by cultivating *Klebsiella oxytoca* No. 19-1 (KCCM 10002) capable of producing cyclodextrin glucanotransferase.

It has been discovered by the present inventors that a newly isolated strain from soil and belonging to genus *Klebsiella* produces a similar enzyme to known one produced by the microorganisms belonging to genus *Bacillus* and *Klebsiella*.

It has been further discovered that the enzyme produced by a newly isolated strain from soil and belonging to genus *Klebsiella* has ability to produce  $\alpha$ -cyclodextrin exclusively from starch.

It has been furthermore found that industrial production of the  $\alpha$ -cyclodextrin can be advantageously accomplished by use of the enzyme since the enzyme produces  $\alpha$ -cyclodextrin in very high proportion from starch, thereby simplify the manufacturing processes of  $\alpha$ -cyclodextrin without an additional gel filtration process or treatment of organic solvent.

The microorganism according to the present invention belongs to genus *Klebsiella* and is identified as *Klebsiella oxytoca*.

This strain was named *Klebsiella oxytoca* No. 19-1 and was deposited with Korean Cultured Center and Microorganism on November 23, 1990 as the deposit number KCCM 10002.

Taxonomical study of the strain was performed according to Bergey's Manual of Systematic Bacteriology and API 20E Kit(France).

Taxonomical characteristics of *Klebsiella oxytoca* No. 19-1 is as follows:

#### 1. Morphological characteristics

Vegetable cell  
: short-rod, 0.3~0.1 $\mu$ m  $\times$  0.6~3 $\mu$ m  
Motility  
: non-motile  
Spore  
: not formed  
Gram staining  
: negative

#### 2. Cultural characteristics

Nutrient agar plate :  
good growth, some protuberance and smoothness, dampness, having colony of lemon yellow.  
Nutrient agar slant :  
good growth, growing on the whole of slant.  
MacConkey agar plate:  
good growth, having colony of red pink color.

#### 3. Physiological characteristics

pH for growth :  
4 ~ 9  
Temperature for growth:  
10~35 °C  
Behavior to oxygen :  
facultative anaerobic  
Hydrolysis of starch  
: positive  
Hydrolysis of casein  
: negative

Hydrolysis of carboxy methyl cellulose  
: negative

Hydrolysis of pullulan  
: positive

5 Liquefaction of gelatin  
: negative

Citrates utilization

: positive

V.P. test

10 : positive

Indole production

: positive

H<sub>2</sub>S production

: positive

15 M.R. test

: negative

Catalase

: positive

Oxidase

20 : negative

Urease

: positive

$\alpha$ -Galactosidase

: positive

25 Reduction of nitrate

: positive

Production of pigments

: negative

Tryptophane desaminase

30 : negative

Lysine decarboxylase

: positive

Ornithine decarboxylase

: negative

35 Arginine dihydrolase

: negative

Fecal coliform test

: negative

40 [Acid production from various sugars ]

Glucose : positive

Mannitol : positive

Inositol : positive

45 Sorbitol : positive

Rhamnose : positive

Sucrose : positive

Melibiose : positive

Amygdalin : positive

50 Arabinose : positive

In accordance with this invention, the process for producing  $\alpha$ -cyclodextrin by using *Klebsiella oxytoca* No. 19-1 isolated from soil as the above mentioned is as follows.

55 The new strain is inoculated in a composited or natural medium and cultivated with shaking.

The medium should consist of starch or amylopectin as a carbon source, and in addition to

that, a nitrogen source and inorganic salts may be used without limit. The strain is cultivated under the aerobic condition in pH adjusted to about 7, and then the culture temperature of 30~40°C is preferred.

Cyclodextrin glucanotransferase from *Klebsiella oxytoca* No. 19-1 reached its maximum activity after 9 hour cultivation and further increase of activity was not observed after that time.

In order to produce  $\alpha$ -cyclodextrin, the enzyme being in the supernatant of culture broth may be used in reaction with starch without purification but it is preferable to use the enzyme purified partially by general method.

Therefore, the present invention provides the efficient process for producing  $\alpha$ -cyclodextrin without any aid of toxic solvents or detergents, and without complicated process like gel filtration to separate  $\alpha$ -cyclodextrin from  $\beta$ - or  $\gamma$ -cyclodextrin in the reaction mixture, owing to the characteristics of the cyclodextrin glucanotransferase produced by *Klebsiella oxytoca* No. 19-1, when starch as a substrate was reacted with an enzyme preparation from cultured medium or partial purification of it under the conditions of pH 7 and 20-55°C.

Following are the examples to illustrate the present invention in further detail but not to limit the scope of the invention.

#### Example 1.

*Klebsiella oxytoca* No. 19-1 was cultivated aerobically in 1L of the medium containing 1% soluble starch, 1% polypeptone, 0.1%  $K_2HPO_4$ , 0.02%  $MgSO_4$ ; 37°C, pH 7, 0.5vvm, 400rpm, 9hr.

After the removal of cells by centrifugation (6000×g, 5 min), supernatant was treated with three volumes of ethanol and maintained at 4°C for overnight.

The formed precipitate was collected by centrifugation (6000×g, 10 min, 4°C), suspended in 50mM phosphate buffer (pH 6), and dialyzed against same buffer for overnight.

Crude enzyme used in this invention was then obtained by lyophilization of the suspension.

The activity of cyclodextrin glucanotransferase was measured according to the method of Lejeune, A. et al. (Analytical Biochemistry, 181, p 6-11, 1989). 1ml of approximately diluted enzyme solution was incubated with 0.6ml of 5% (w/v) soluble starch, 0.105ml of 1mM methyl orange and 1.295ml of 50mM phosphate buffer (pH 6) at 37°C for 10 min.

The reaction was ceased by addition of 0.150ml of 6N HCl and maintained at 15°C on a water bath for 30 min.

By determination of optical density at 507nm, the activity of the enzyme could be calculated with

the prepared standard curve.

One unit of the enzyme activity was defined as the amount of enzyme which produces 1 $\mu$ mole of  $\alpha$ -cyclodextrin per minute under the given conditions.

#### Example 2.

10% (w/v) soluble starch solutions was prepared in 1 liter of 50mM phosphate buffer (pH 7) and 1mM  $CaCl_2 \cdot 2H_2O$  and solubilized by autoclave for 5 min.

After cooling the solution, 0.5g of crude enzyme (1700 units) prepared by the above example 1 was added to the solution.

The reaction mixture was then incubated at 40°C for 20hr with stirring and boiled on a water bath for 5 min to inactivate the enzyme.

Profile and content of cyclodextrins in the reaction mixture were determined by HPLC under the following conditions: Carbohydrate column (Waters Co., USA), RI detector, acetonitrile/water (65/35), flow rate 2.0 ml/min.

Before the analysis, the reaction mixture was treated with equal volume of eluent and filtered through a membrane (0.45  $\mu$ m) to remove remaining substrate.

As shown in Fig 1, high amount of  $\alpha$ -cyclodextrin was only detected and other cyclodextrins were not observed.

The estimated concentration of  $\alpha$ -cyclodextrin was 15g per 100g of starch when compared with standard cyclodextrins.

This result means that high amount of  $\alpha$ -cyclodextrin is produced with a very high selectivity in a short reaction time without any aid of toxic solvent or detergent to increase the ratio of  $\alpha$ -cyclodextrin in reaction mixtures.

Therefore, highly purified  $\alpha$ -cyclodextrin product was easily prepared by concentration and drying processes from  $\alpha$ -cyclodextrin in the reaction mixture and consequently, it was possible to eliminate the complicated process like gel filtration in manufacturing processes of  $\alpha$ -cyclodextrin.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a result of HPLC analysis for cyclodextrin reactant obtained after reaction for 20 hours according to example of this invention.

Fig. 2 is a result of HPLC analysis for the known standard  $\alpha$ -,  $\beta$ -, and  $\gamma$ -cyclodextrin produced on a commercial basis.

#### Claims

1. Klebsiella oxytoca No. 19-1 (Deposit No. ; KCCM 10002) having the ability to digest starch and to produce  $\alpha$ -cyclodextrin with a very high selectivity from starch.

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2. A process for producing  $\alpha$ -cyclodextrin exclusively by reacting starch as a substrate with an enzyme preparation selected from cultured medium which is obtained by cultivating microorganism, Klebsiella oxytoca No. 19-1 capable of producing cyclodextrin glucanotransferase.

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Fig.1

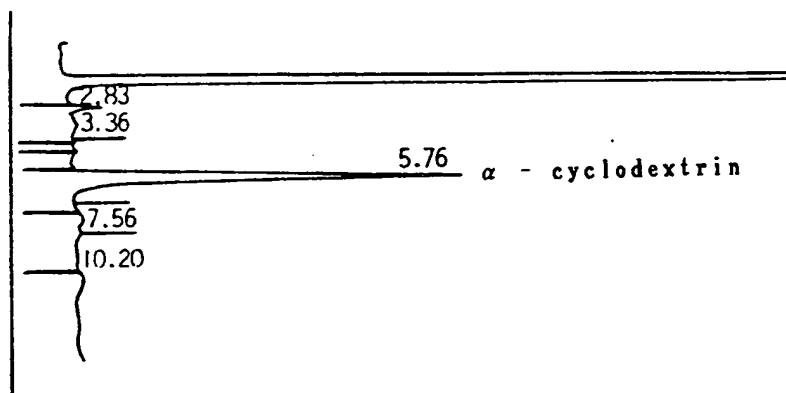
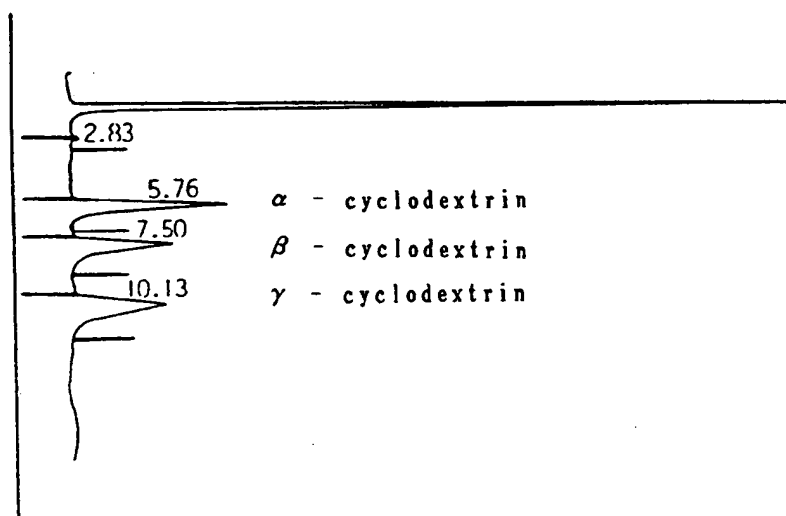


Fig.2





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## EUROPEAN SEARCH REPORT

Application Number

EP 91 12 1755

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	PATENT ABSTRACTS OF JAPAN vol. 13, no. 549 (C-662)(3897) 7 December 1989 & JP-A-1 225 493 ( NAKANO VINEGAR CO LTD ) 8 September 1989 * abstract *	1,2	C12N1/20 C12P19/18 //(C12N1:20; C12R1/22)
A	WO-A-8 901 043 (GENETICS INSTITUTE INC.) * page 4, last paragraph - page 7, last paragraph *	1,2	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
			C12P C12R
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 19 FEBRUARY 1992	Examiner MONTERO LOPEZ B.
<b>CATEGORY OF CITED DOCUMENTS</b> X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons * : member of the same patent family, corresponding document			

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